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Review

Genetic mechanisms of postzygotic reproductive isolation: An epistatic network in rice

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Products of interspecific crosses often show abnormal phenotypes such as sterility, weakness and inviability. These phenomena play an important role in speciation as mechanisms of postzygotic reproductive isolation (RI). During the past two decades, genetics studies in rice have characterized a number of gene loci responsible for postzygotic RI. I have identified 10 loci including three sets of epistatic networks in a single intersubspecific cross (*Oryza sativa* ssp. *indica* × *japonica*). These results suggest that RI genes cause developmental dysfunction of vegetative and/or reproductive organs through a variety of molecular pathways. The latest molecular studies demonstrated that hybrid incompatibility is mainly due to deleterious interactions caused by species-specific mutations of two or more genes, mediated by proteins acting within the same molecular pathway. Because genetic interactions provide a perspective on gene function, epistatic networks are a key to the understanding of the molecular basis of postzygotic RI. In this review, I focus on recent progress in postzygotic RI studies in rice and discuss the evolutionary significance as well as implications for improving rice productivity.

Key Words: reproductive isolation, hybrid sterility, epistasis, rice, *indica*, *japonica*.

Introduction

The mechanisms of speciation are a central problem in biology. The development of prezygotic and/or postzygotic reproductive isolation (RI) are key steps in speciation. Genetic postzygotic RI mechanisms are categorized into F_1 hybrid incompatibility and hybrid breakdown of F_2 or later generations. Most cases of postzygotic RI are genetically controlled, and genetic and molecular biological studies have been conducted in a wide variety of animals (Mouse: Bauer *et al.* 2005, Drosophila: Phadnis and Orr 2009, Wu *et al.* 1988) and plants (Ichitani *et al.* 2007, Koide *et al.* 2008, Rieseberg *et al.* 1996, Sweigart *et al.* 2006, Taylor *et al.* 2009). Since F_1 hybrid incompatibility arises in the heterozygous condition while hybrid breakdown requires the recessive homozygous condition for a part of the complementary genes, the molecular mechanisms underlying the two phenomena should be different. For example, the causal recessive gene in hybrid breakdown evokes a loss-of-function mutation of genes essential for normal development, whereas a deleterious heterodimer structure of the causal proteins or a negative interaction between different molecules such as DNA/RNA and proteins are thought to be involved in the F_1 incompatibility mechanism. Little is known, however, about the difference on the molecular level, because of our limited understanding of the processes of F_1 incompatibility and hybrid breakdown. Elucidating the causal genes and molecular mechanisms involved in the various types of postzygotic RI will have broad implications for evolutionary biology, reproductive and developmental systems, and the improvement of domesticated animals and plants.

Rice belongs to the genus *Oryza*, which consists of 20 wild and two cultivated species. Asian cultivated rice, *Oryza sativa* L., was domesticated from the wild species, *O. rufipogon*, which together are referred to as the "*sativa-rufipogon* complex". *Oryza rufipogon* is widely distributed across Asia to Oceania. The exposure to such a wide spectrum of environmental conditions endowed the *sativa-rufipogon* complex with an extensive range of ecological, morphological and physiological characters of adaptive significance. The complex therefore represents a reservoir of genes conferring resistance against biotic and antibiotic stresses and genes that control yield components. Due to different morphological and physiological characters, *O. sativa* is divided into

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two subspecies, *indica* and *japonica* (Kato 1930). Recent phylogenetic and comparative genome studies demonstrated that *indica* and *japonica* were domesticated independently from multiple subpopulations of *O. rufipogon* (Huang *et al.* 2012, Londo *et al.* 2006, Wei *et al.* 2012). With this evolutionary history including different types of ecological adaptation and subsequent domestication, the *indica* and *japonica* subspecies have developed partial postzygotic RI. F_1 hybrid sterility is the most commonly observed mechanism of postzygotic RI in *indica/japonica* crosses. So far, more than 40 genes have been reported to be involved in hybrid sterility in rice (Supplemental Table 1). Classical genetic studies proposed two genetic models for F_1 hybrid sterility, the interlocus epistasis model also referred to as the Bateson-Dobzhansky-Muller (BDM) model (Bateson 1909, Dobzhansky 1937, Muller 1942), and the one-locus allelic interaction model also known as sporogametophytic interaction (Ikehashi and Araki 1986, Rick 1966, Sano 1990). Recent molecular genetic studies of the one-locus model specified the unique properties of its molecular structures. Comparing the classical genetic models, this review focuses on new findings from genetic, molecular and evolutionary studies on postzygotic RI in rice. The mechanisms of F_1 hybrid sterility and hybrid breakdown will be described separately, and methodologies for dissecting RI genetic networks and applications in plant breeding will be discussed.

Allelic interactions responsible for F_1 hybrid sterility

There are numerous studies reporting F_1 hybrid sterility in *indica/japonica* crosses (Supplemental Table 1). Most of the occurrences, with a few exceptions, were explained by allelic interaction at a single locus. "Gamete killer" is a typical case of the one-locus model (Rick 1966). Three different alleles, killer (S^k), abortive (S^a) and neutral (S^n), comprise the gamete killer system. Gametes carrying the S^a allele are abortive in heterozygous hybrids, while gametes carrying S^k are fertile in S^k/S^a heterozygous plants. The neutral allele (S^n) generates fertile heterozygous hybrids either with S^k or S^a homozygous parents. Many rice researchers have supported this genic model in their studies, and have applied the triallelic system to newly identified genes. Recently, cloning and characterization of the *S5* genes unraveled the functioning of the triallelic system at the molecular level (Yang *et al.* 2012). The *S5* locus contains three genes tightly linked within a 50-kb region and deleterious allelic combinations of these three genes causes female gamete abortion via endoplasmic reticulum (ER) stress induction in *indica-japonica* hybrids. Before this study, another hybrid male sterility gene, *Sa*, was also found to interact with two adjacent genes encoding an F-box protein and E3 ligase (Long *et al.* 2008). These two reports clearly demonstrated that two or more tightly linked genes form functional complexes and that heterozygous polymorphic gene complexes lead to gamete abortion in hybrid plants. Thus, a part of the genetic architecture of the one-locus model is a cluster of functionally related genes that are inherited together like single genes due to their tight linkage. This genetic mechanism substantially corresponds to the BDM model. Another aspect of the one-locus model was revealed in a study of the hybrid sterility gene *S24*. The mode of inheritance of *S24* fits well the one-locus allelic interaction model, as male gametes carrying the *indica* allele, *S24-i*, are more frequently transmitted than those carrying the abortive *japonica* allele, *S24-j*, in *S24* heterozygotes, while homozygotes for *S24* are fully male fertile (Kubo *et al.* 2008). Like in the cases of *S5* and *Sa*, *S24* sterility is heterozygous-specific. Kubo *et al.* (2011) showed that *S24* sterility depends on an unlinked gene named *EFS* (*Epistatic Factor for S24*) (Fig. 1). *S24* is suppressed in the presence of a dominant *indica* allele of *EFS* (*Efs-i*), resulting in good fertility, whereas *S24* is activated and causes male semi-sterility only in plants homozygote for the recessive *efs-j*. Thus, epistatic regulation is essential for allelic interactions of hybrid sterility genes, a phenomenon I called "epistasis-based allelic interaction". *S35*, another hybrid male sterility gene, was also demonstrated to be under epistatic control (Kubo *et al.* 2008). The *S24-i* allele is necessary for *S35* to cause male sterility, and *S35-j*-bearing male gametes are abortive in an *S35* heterozygous context. Koide *et al.* (2012) also suggested an involvement of an interaction with unlinked modifiers for the gamete eliminator gene *S6* which was found in an *O. sativa/O. rufipogon* cross. Combined with the other cases (*S5* and *Sa*), these findings suggested that one-locus allelic interaction systems might be specific, complex versions of the BDM interaction, formed by hierarchic components representing killer-protector (Yang *et al.* 2012), killer-modifier (Koide *et al.* 2012, Sano 1990) and killer-killer interactions (*S24-S35* interaction in Kubo *et al.* 2008). In previous studies on hybrid sterility in animals, complex networks of clusters of functionally related genes and unlinked genes have been reported [e.g. segregation distorter in Drosophila by Wu *et al.* (1988), t-complex in mouse by Bauer *et al.* (2005)]. These facts suggest a structural commonality between animals and plants with regard to the development of postzygotic RI mechanisms. Consequently, F_1 hybrid incompatibility appears generally controlled by epistatic networks involving multiple genes and seems to be controlled rarely by a single gene. The discovery of inconsistent phenotypes of *S24* heterozygotes in *japonica* and *indica* genetic backgrounds led to the identification of *EFS* (Kubo *et al.* 2011, see also Fig. 2). In a similar fashion, the killer-type gene *S25* allowed for complete male fertility in the *indica* background (unpublished data), but induced semi-sterility in the *japonica* genetic background (Win *et al.* 2009). This result suggests that *S25* also is epistatically controlled by an unlinked gene which has not been identified yet and supports the "epistasis-based allelic interaction" model for F_1 hybrid sterility. Thus, epistasis generally is involved in what conventionally are assumed to be one-locus allelic interactions.

A different genetic mechanism of F_1 hybrid sterility is the loss of function in duplicate genes that was first demonstrated

by senior rice geneticist Oka (1974). Cloning and sequencing studies revealed that loss-of-function mutations of duplicate genes cause functional defects in male gametes in rice (Mizuta *et al.* 2010, Yamagata *et al.* 2010). Potentially plant F_1 hybrid incompatibility could be explained by simple genetic mechanisms involving a small number of loci, such as one-locus allelic interactions or duplicate genes. However, the studies cited above indicate that both genetic models include epistasis-related effects among multiple linked or unlinked loci. It is expected that diversified forms of epistatic interactions will be revealed by future cloning studies of additional F_1 sterility genes.

The three F_1 sterility genes, *S24*, *S25* and *S35*, cause developmental defects at mitotic stages of male gametogenesis (Kubo *et al.* 2008, Win *et al.* 2009). Interestingly, other rice F_1 sterility genes tend to evoke developmental defects at late stages of gametogenesis, while genes that do so at earlier and meiotic stages are rare. The gametophytic sterility due to defects in late gametogenesis has a lesser effect on seed fertility than the sporophytic sterility due to premeiotic and meiotic defects. It is unclear which components of the mechanism lead to such bias, but it is known that the strength of reproductive barriers is proportional to the genetic distance between two species (Coyne and Orr 1989, 1997, Moyle *et al.* 2004, Presgraves 2002). Actually, there are no cases of F_1 hybrid inviability in intraspecific crosses of *O. sativa*, but interspecific crosses among cultivated and wild rice species have been reported to show F_1 inviability (Chu and Oka 1970). Thus, the reproductive isolation is incomplete between *indica* and *japonica.*

Complementary genes for hybrid breakdown

Hybrid breakdown is defined as sterility or weakness observed in the F_2 or later hybrid generations while the F_1 hybrids grow normally with good fertility. In general, fewer case studies of hybrid breakdown than of F_1 hybrid incompatibility have been published and therefore the molecular basis of hybrid breakdown remains obscure. The genetics of hybrid breakdown have been studied in rice (Fukuoka *et al.* 1998, Yamamoto *et al.* 2010) and a simple genetic mechanism based on duplicate recessive genes (15 : 1 segregation in F_2) has been identified. Examples are the gene pairs *hwe1* and *hwe2* (*hybrid weakness-e-1* and *-e-2*) (Kubo and Yoshimura 2002, see also Fig. 1) and *hbd2* and *hbd3* (*hybrid breakdown 2* and *-3*) (Yamamoto *et al.* 2010). The double recessive homozygote for *hwe1* and *hwe2* causes poor vegetative growth and complete sterility, but the molecular basis has not yet been elucidated. On the other hand, *hbd2* and *hbd3* were found to encode casein kinase I and NBS-LRR, respectively, and the hybrid breakdown was attributed to an autoimmune response (Yamamoto *et al.* 2010). Similarly, F_1 hybrid necrosis in Arabidopsis, tomato, and lettuce, was found to be due to epistatic interactions between pathogen resistance genes (Alcazar *et al.* 2010, Kruger *et al.* 2002, Jeuken *et al.* 2009). These findings indicated a link between immune response systems and postzygotic RI development in plant evolution. In other cases, complex interactions between three complementary genes occur in *indica/japonica* crosses (Kubo and Yoshimura 2005). The three genes, *hsa1*, *hsa2* and *hsa3* (*hybrid sterility-a-1*, *-2* and *-3*), which are located on rice chromosomes 12, 8 and 9, respectively (Fig. 1), showed different inheritance patterns in segregating populations. The recessive gene *hsa1* causes sporophytic sterility and sterility segregates at a 3 : 1 ratio in selfed progeny of the *hsa1* heterozygotes. On the other hand, *hsa2* and *hsa3* cause gametophytic sterility (sterility phenotype determined by gamete genotype) resulting in segregation distortion (nearly equal to a 0.1 : 1 : 1 ratio deviating from the expected 1 : 2 : 1 ratio). Because the *hsa1* gene is recessive, interaction of these genes has no significant effects on F_1 hybrid phenotypes, which is why this sterility phenotype is a case of hybrid breakdown. Although the molecular basis remains unknown, the different inheritance modes of the three genes suggest an interaction between different molecules such as different protein family members or DNA/RNA, rather than duplicate genes encoding a single protein.

Together with epistasis-based allelic interactions, a variety of other epistatic interactions seem to contribute to postzygotic RI. Many RI genes, which were found in genome-wide surveys including QTL analysis and CSSL analysis, have been detected in a variety of cross combinations between different rice cultivars and species. In contrast, through all my previous studies (Kubo and Yoshimura 2001, 2002, 2005, Kubo *et al.* 2008, 2011, Win *et al.* 2009), a total of 10 major gene loci responsible for postzygotic RI have been found in a single cross between Asominori and IR24 (Fig. 1). More recently, my data is suggesting that several genes relating to this genetic network still remain to be identified. Because all these genes were identified in an intraspecific cross, an equal number or more genes should be expected to be involved in postzygotic RI of crosses between more remotely related parents. However, there are no reports of similar gene numbers in other cross combinations, suggesting that we have identified only a very small fraction of the genes involved in plant postzygotic RI. It is intriguing that such a large number of RI genes have developed at subspecies level and how they may have contributed to rice speciation.

Toward a better understanding of a complex genetic network

Epistasis has become a central genetic concept in understanding postzygotic RI as well as other quantitative traits. Despite this importance, epistasis has been elusive. Among several types of epistasis, digenic interaction has often been observed as digenic segregation patterns like 15 : 1 or 11 : 5 (Fukuoka *et al.* 1998, 2005, Kubo *et al.* 2002, Yamamoto *et al.* 2010). However, there are few studies that have characterized multiple-gene interactions with more than two unlinked loci. Furthermore, we have never established exactly

how many genes were involved in individual incompatible phenotypes. Almost all cases of postzygotic RI studies have focused on only one or two genes/chromosome region(s) that evoke a major phenotypic effect, and have ignored other unlinked interacting or cryptic factors with minor effects in a given genetic background. The reason is that dissecting complex genetic networks requires hard work and a lot of time. How can such networks be untangled?

Chromosome segment substitution lines (CSSLs) have been developed in many plant species to facilitate the discovery of genes/QTLs responsible for natural variation (Eshed and Zamir 1995, Kubo *et al.* 2002, Ramsay *et al.* 1996). To date, CSSL have proven to be a powerful tool for the positional cloning of genes, and the evaluation of gene-gene as well as gene-environment interactions for many types of traits (Doi *et al.* 2004, Wei *et al.* 2010, Yu *et al.* 2007). Multiple concurrent introgressions produced by intercrossing different CSSLs provide an important source of segregating populations to evaluate multiple-gene interactions. Additionally, reciprocal sets of CSSL series are useful for solving the complex network problem and enable us to specify the number of participant genes in a network. If a sterility phenotype is masked in a certain genetic background, we may postulate the existence of additional interacting genes (Fig. 2). Conversely, if the same phenotype is consistently observed in two alternative genetic backgrounds, we may conclude that the genes under study represent the complete set, and no more genes may be required to control the sterility phenotype. Actually, this step-by-step approach using reciprocal CSSLs already has led to the identification of two sets of epistatic networks, namely *hsa1–has2–hsa3* and *EFS–S24–S35* (Kubo and Yoshimura 2005, Kubo *et al.* 2011).

Gene clusters putatively related to *indica-japonica* differentiation

The effects of adaptive evolution on gene sequences, gene expression levels and the resulting molecular pathways, and how RI between divergent populations develops in this evolutionary process are questions of general biological interest. A tendency toward linkages between major morphological genes and RI genes has been observed in the analysis of series of CSSLs. For example, the replacement of a chromosome segment in the Asominori genome by the corresponding segment from the cultivar IR24 that contained the RI genes *S24* and *S35*, introduced IR24-like morphological traits including narrow grains and increased number of grains per panicle (Fig. 3). These morphological changes probably were caused by the genes *qSW5/GW5* for grain width (Shomura *et al.* 2008, Weng *et al.* 2008) and *Gn1a* for grain number per panicle (Ashikari *et al.* 2005), because of the similar positions on the chromosomes. Additionally, *S24* was very tightly linked to another hybrid female sterility locus, *S31* (Zhao *et al.* 2007). Both *qSW5/GW5* and *S31* were identified using CSSLs derived from Asominori and IR24 (Weng *et al.* 2008, Zhao *et al.* 2007). Other RI genes also tend to tightly or loosely link with major trait genes such as heading date (*Hd3a*, *Hd1* and *S26* on chromosome 6, *DTH8* and *hsa2* on chromosome 8) (Kojima *et al.* 2002, Kubo and Yoshimura 2001, Yano *et al.* 2000, Wei *et al.* 2010) and blast resistance and brown planthopper resistance genes (*Pi-ta* and *hwe1*, *Bph26* and *hsa1* on chromosome 12) (Bryan *et al.* 2000, Yara *et al.* 2010). Among 10 RI genes mapped on rice chromosomes (Fig. 1), eight were linked with some major trait gene(s). Traits such as heading date and biotic stress resistance probably had a significant role in rice evolution. The linkages between incompatibility genes and favorable trait genes likely reflect a hitchhiking effect associated with natural selection and/or domestication. These gene clusters appear related to the *indica-japonica* differentiation and

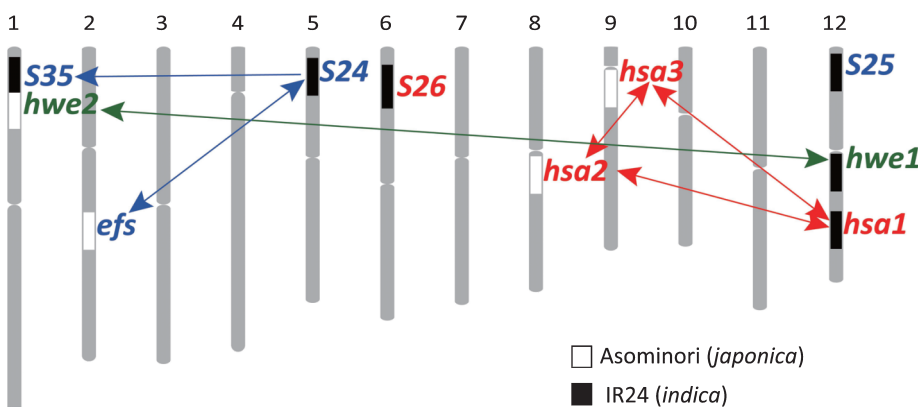

Fig. 1. Chromosome map showing the genetic network underlying postzygotic RI in an Asominori (*japonica*) × IR24 (*indica*) cross. Colored letters give the phenotypic classification connected to a locus; deleterious allelic combinations are shown as black (IR24 allele) and white (Asominori allele) boxes. Mutual and unidirectional gene interactions are indicated by double-headed and single-headed arrows, respectively. Blue letter: Male sterility gene, Red letter: Female sterility gene, Green letter: Weakness gene.

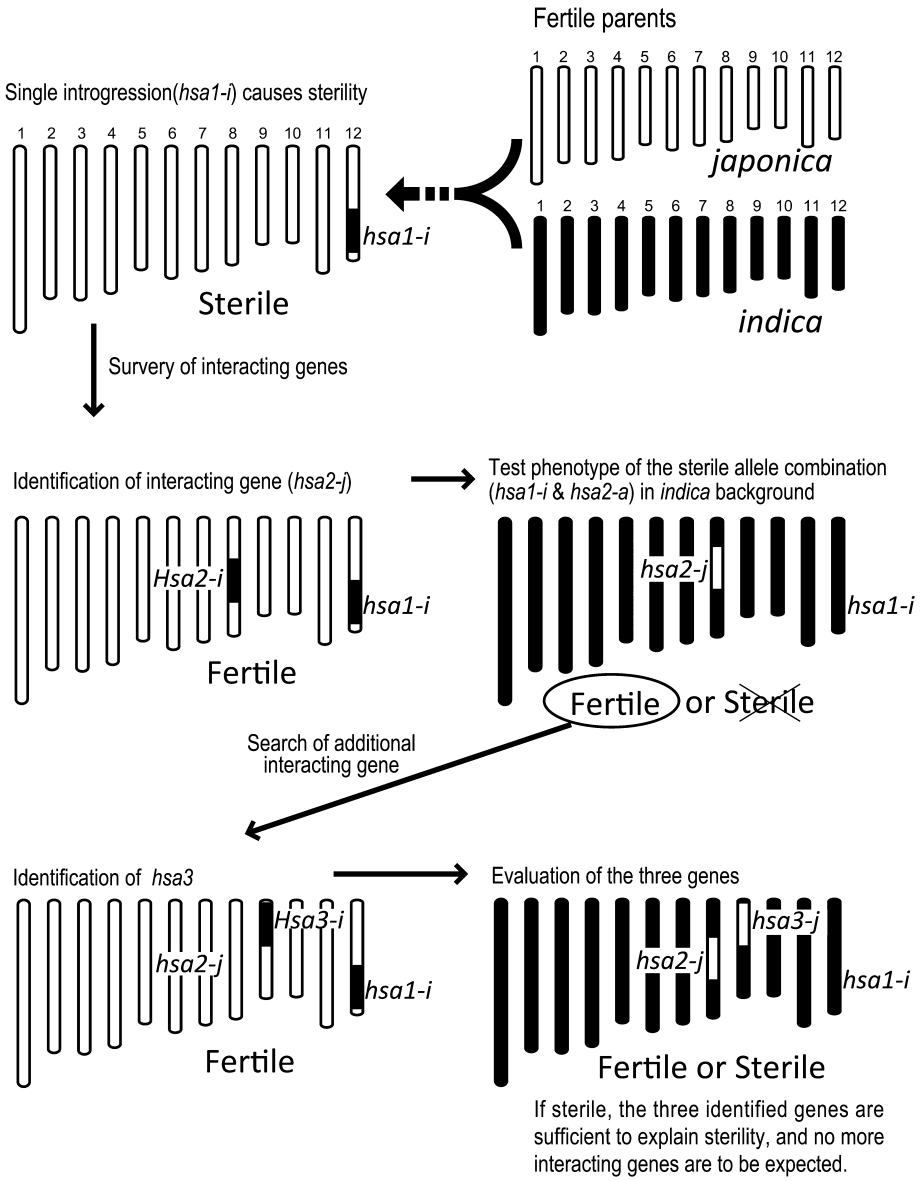

Fig. 2. Dissecting the epistatic network by using reciprocal CSSLs. The example of the three complementary genes, *hsa1*, *hsa2* and *hsa3* is used; the gene set of *hsa1-i hsa2-j hsa3-j* is incompatible and causes high seed sterility. Once a digenic interaction is identified, it is necessary to confirm the resulting sterile phenotype in the alternative genetic background. If the same phenotype is observed, the identified genes probably represent the complete gene set causing sterility. If not, additional interacting gene(s) can be expected (in this case, a restorer is hidden in the *indica* background). *hsa1-i* and *hsa2-j* denote the *indica* allele of *hsa1* and the *japonica* allele of *hsa2*, respectively. For details, refer to Kubo and Yoshimura (2005).

therefore should become a focus of molecular studies into rice evolution and domestication. Similarly, linkages between morphological genes and viability genes (referred to as "M-V linkage") has been observed in several plant species, as reviewed by Grant (1967). Because M-V linkages in plants are responsible for linkage drag, their knowledge is critical to crop breeding.

Conclusions and future perspective

Molecular genetics studies in rice have revealed that clusters of adjacent interacting genes and epistatic interactions are involved in the mechanism of F_1 hybrid sterility. These studies suggest that the gradual accumulation of mutations of functionally related genes promoted the development of RI in the evolution of diverging populations. Consequently, the genetic network underlying RI evolution appears more complex than generally thought. The causal molecular pathways are likely to be different in each case as molecules of different functions are involved, such as heat shock protein and aspartic protease (Yang *et al.* 2012), E3-like ligase and F-box protein (Long *et al.* 2008), mitochondrial L27 protein

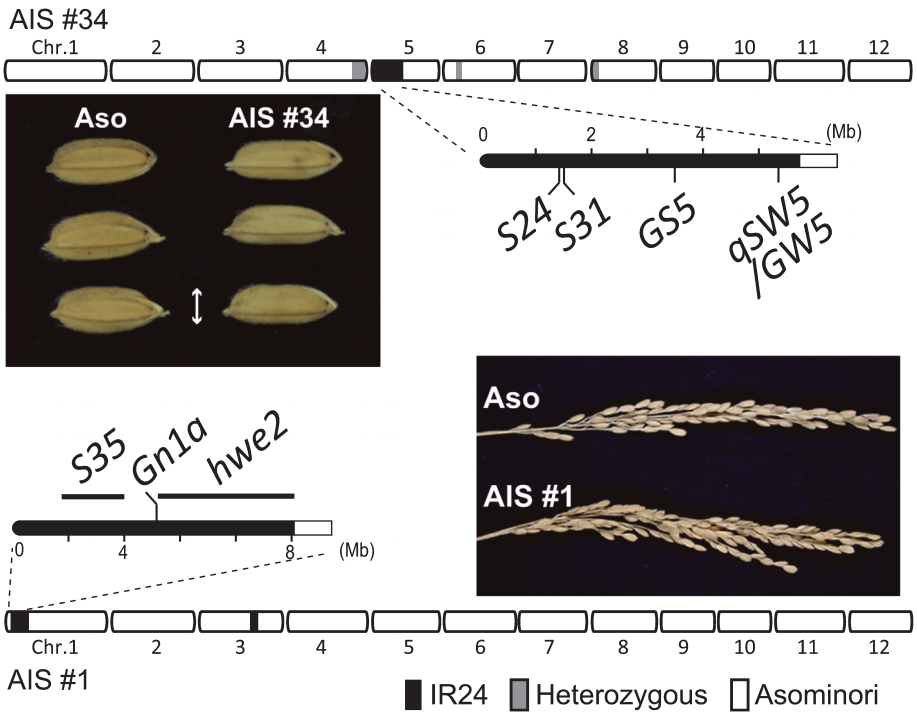

Fig. 3. Chromosome maps showing gene clusters containing RI and morphological genes on the short arms of rice chromosomes 1 and 5. Photographs demonstrate the morphological difference between cultivar Asominori (recipient) and the CSSL (AIS) that had received IR24 chromosome segments containing the RI genes. AIS #1 showed increased grain numbers per panicle compared to Asomiori [Asominori: 88.3 ± 6.3, AIS #1: 147.0 ± 12.0 (Means ± SD)]. AIS #34 showed reduced grain width compared to Asomiori [Asominori: 3.49 ± 0.01 mm, AIS #34: 3.21 ± 0.03 mm). For comparison, grain number and grain width of IR24 (donor) were 190.9 ± 34.1 and 2.91 ± 0.01 mm, respectively. *GS5* on chromosome 5 also regulates grain width (Li *et al.* 2011), but it remains unknown whether *GS5* contributes to the grain size variation between Asominori and IR24. Aso: Asominori, AIS: CSSL carrying IR24 segments in the Asominori genetic background.

(Yamagata *et al.* 2010) and casein kinase and NBS-LRR (Yamamoto *et al.* 2010). My previous studies have revealed various types of incompatibility phenotypes and genetic mechanisms even in a single cross of *indica* and *japonica* rice. It is worth noting that there is no report, verified by molecular analysis, of an RI gene that is sufficient to cause hybrid incompatibility by itself in any plant species. Since most postzygotic RI traits are due to defects in reproductive development, the study of functional molecules and their networks that control reproductive development will foster a better understanding of the mechanisms of RI. The molecular pathways of plant reproductive development have remained largely unknown, but the genes and a part of the molecular networks have become clearer in recent years through mutant and transcriptome analyses (Aya *et al.* 2011, Fujita *et al.* 2010, Kubo *et al.* 2013, Li *et al.* 2011, Moon *et al.* 2013, Tan *et al.* 2012). Coupled with new findings in reproductive development, future analyses will elucidate the molecular basis of RI and reproductive systems in plants.

Plant breeders have long been facing the challenge of developing high yielding and stress-tolerant varieties. Because the rice germplasm including its wild relatives presents a wide genetic diversity and sources of heterosis, hybrid rice breeding has a large potential for the improvement of yield, stress tolerance and other agronomical traits. However, the actual utilization of the rice germplasm has been limited to combinations of elite varieties that lack sterility barriers. Understanding RI genes and their molecular function will help to remove this limitation. Physical mapping of RI genes will facilitate the breakage of linkage drag by marker-assisted selection. In addition, the understanding of epistatic interactions can be utilized to mask the harmful effects of RI genes.

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